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NODAL AND TERMINAL SPROUTING FROM MOTOR NERVES IN FAST AND SLOW MUSCLES OF THE MOUSE

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SUMMARY

1. A study of nodal and terminal sprouting in fast and slow muscles of the mouse hind limb has been made using the zinc iodide and osmium tetroxide stain.

2. The terminal sprouting normally elicited by botulinum toxin injection can be prevented by regular and frequent direct electrical stimulation of the muscle fibres. But the number of end-plates innervated by nodal sprouts in partly denervated spinal preparations was not reduced by direct muscle stimulation.

3. In leg muscles given varying doses of botulinum toxin the amount of terminal sprouting was linearly related to the degree of paralysis. In partly denervated muscles neither the amount of terminal sprouting nor the amount of nodal sprouting was correlated with the degree of denervation.

4. Partial denervation causes relatively more nodal sprouting in the fast muscles peroneus tertius and extensor digitorum longus than in the slower soleus muscle, which itself has considerably more terminal sprouting than the others. The fast muscles can develop as much terminal sprouting as the soleus only in response to full paralysis with botulinum toxin.

5. No evidence could be found for a sprouting signal generated or spreading within the spinal cord.

6. It is concluded in confirmation of earlier work (Duchen & Strich, 1968; Brown & Ironton, 1977*a*) that the source of the signal for terminal sprouting is denervated or otherwise inactivated muscle fibres, whose action is boosted by the presence of degenerating nervous tissue. It is suggested that fast muscles probably have less terminal sprouting when partly denervated than slow muscles (*a*) because of the longer time it takes a fast muscle to undergo the changes associated with inactivity and (*b*) because of their higher resistance to the effects of nerve degeneration. It does not seem that the signal for nodal sprouting comes from the muscle fibres but further experimentation is needed to establish this firmly.

INTRODUCTION

Hoffman (1950) in his excellent original description of sprouting in motor axons of partly denervated rat muscles observed two forms of sprouting from the intact axons: growth from the terminals themselves and growth from nodes of Ranvier near the axon terminal. He also analysed various possible sources for the stimulus that evoked

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this reaction and concluded (Hoffman, 1950; Hoffman & Springell, 1951) that as axon and myelin broke down at the peripheral end of the nerve they released a fatty acid which was able to insert itself in the terminal and nodal membrane and, in some way unknown, provoke the growths from these two sites.

Since then it has become clear that muscle paralysis, which it is now known can bring about changes in mammalian muscle almost identical to those seen on denervation (Lømo & Rosenthal, 1972; Lømo & Westgaard, 1976) can by itself bring about sprouting (Duchen & Strich, 1968; Brown & Ironton, 1977*a*; Holland & Brown, 1980). However, the sprouting appears at the light microscope level at any rate, to be only from terminals (Duchen & Strich, 1968; Ironton, Brown & Holland, 1978). More recently still it has been suggested that in the frog a further stimulus to sprouting may arise within the central nervous system, spreading from axotomized and chromatolysing motoneurones to intact ones (Rotshenker, 1979).

In this paper we have analysed the different patterns of terminal and nodal sprouting that occur in different muscles in response to several different experimental manipulations. The results support the hypothesis that changes in the muscle fibres associated with denervation or inactivity are the stimulus for terminal but probably not nodal sprouting. Evidence is presented that terminal sprouting is not due to failure of delivery of nerve-borne antisprouting agents as postulated to occur in salamander cutaneous nerves by Diamond, Cooper, Turner & Macintyre (1976). In addition no evidence could be found for there being a central stimulus involved in initiating sprouting in mice.

Some of these results have been presented in brief and abstract form as Communications to the Physiological Society over the last three years (Brown, Goodwin & Ironton, 1977; Brown, Holland & Ironton, 1978*a*, *b*, 1979).

METHODS

All the experiments were performed on male and female adult white mice of the Carworth CFLP, and OLAC strains weighing 20–45 g. The muscles used were the soleus, the peroneus tertius, and the extensor digitorum longus (e.d.l.). The operations were initially carried out under ether anaesthesia (with a pre-medication of 0·3 ml. of 1 μg/ml. atropine + 0·3 ml. of 0·1 μg/ml. propanolol). More recently at the suggestion of Drs A. Östberg and G. Vrbova, we have used chloral hydrate as the anaesthetic (0·2 ml. of 3·5 mg/ml. per 10 g body wt) with atropine as a premedication (0·3 ml. of 1 μg/ml.).

Partial denervation. This was usually performed by wholly or partly cutting through the L4 or L5 sciatic rami. It is worth noting that the variable distribution of motor axons to soleus and peroneus tertius between these roots fairly frequently led to muscles being either completely denervated or not denervated at all.

The rami were exposed either by a ventral operation as described before (Brown & Ironton, 1978) or by approaching dorsally, removing the lumbar muscles from their vertebral attachments until the ramus was visible between two adjacent transverse processes. For L4 section, the ramus was exposed between the L5 and L6 transverse processes, as it emerges from under the L5 process. The ramus was cut using fine scissors. Alternatively, partial denervation was achieved by cutting part way through the sciatic trunk in the thigh region, though this method proved even less consistent.

Spinalization. The cord was approached dorsally, the muscles attached to the vertebrae in the high lumbar region were freed, and the junction between the L1 and L2 vertebrae exposed. These vertebrae were then eased apart, and the cord was either cut through using scissors or the tip of a diathermy was inserted and heat was applied for a few seconds.

Chronic muscle stimulation. The soleus and peroneus tertius muscles were electrically stimulated using the bared ends of Teflon-coated multistranded stainless steel wires (Bioflex AS 632 Cooner Sales Co. California) inserted under the skin on either side of the leg. The wires, held in place by silk sutures, were led under the skin to the base of the tail and along the tail, being brought to the surface through a small nick in the tail skin. They were connected to a stimulator delivering 100 μsec pulses of 20–40 mA amplitude at 100 Hz for 1s every 100 sec. The direction of current flow reversed after every pulse to prevent electrode polarization. This pattern is particularly effective in inhibiting the changes which occur in denervated muscle fibres (Lømo & Westgaard, 1976).

Injections. These were made into the back of the lower hind limb just above the achilles tendon, using a 10 μl Hamilton syringe. Various doses of botulinum toxin, crude type A, were used - see results section for details.

The unpurified botulinum toxin was kindly supplied by Dr Melline of M.R.E. - Porton, LD_{50} for 20 g mouse = 0·004 μg, I.P. It was dissolved in phosphate buffer (pH = 6·6).

Acute experiments. The muscles were examined *in vitro* essentially as described by Brown & Ironton (1978). The amount of neuromuscular block or partial denervation was assessed by comparing the direct and indirectly evoked twitch and tetanic tensions. The amount of denervation was also measured by grading the stimulus to the nerve and counting the number of discrete levels of tension which gave a measure of the number of motor units present.

The extrajunctional ACh sensitivity of the muscle was measured as the contracture produced by perfusing the muscle in 5×10^{-4} g/ml. ACh perchlorate and expressing this as a fraction of the maximal 50 Hz directly evoked tetanic tension. A normal soleus and peroneus will produce a contracture of about 5% of the tetanus. A completely denervated or blocked muscle produces a contracture of about 70%.

Histology. The motoneurone terminals and intramuscular branches were stained with zinc iodide and osmium tetroxide (ZIO), as described by Brown & Ironton (1978).

At least fifty end-plates from each muscle were examined. The end-plates measured were effectively randomly chosen as the muscle had been teased into small bundles of fibres (ca. 20) which were scattered on the slide. All the end-plates in each bundle looked at were studied. The lengths of the end-plate and any terminal sprouts were measured along the long axis of the innervated muscle fibre. Examination of the slides was always done, 'blind', where possible, with the observer not knowing the experimental history of the muscle. On many occasions, 'blind' examination was carried out by more than one of the authors, and very good agreement has always been found. Our criteria for terminal sprouting have been conservative in that we only classify a terminal as having a sprout or sprouts if there is a clear fine outgrowth(s) breaking up the normal smooth end-plate outline (see Pl. 1). End-plates were classified as being supplied by terminal sprouts if the unmyelinated branch running into the end-plate could be traced back to another end-plate supplied by a myelinated axon. End-plates which were supplied by an unmyelinated axon which ran a long, often rather straight course from a group of myelinated axons were classified as being supplied by nodal sprouts (see Pl. 1).

RESULTS

Reduction of terminal sprouting during botulinum toxin poisoning by direct muscle stimulation

The extensive terminal sprouting originally described by Duchen & Strich (1968) which occurs in botulinum toxin poisoning might be due simply to inactivity-dependent changes in the muscle (Lømo & Rosenthal, 1972) or to blockage of release of an antisprouting agent (Diamond *et al.* 1976). Duchen, Rogers, Stolkin & Tonge (1975) and Duchen & Tonge (1977) have shown that an additional muscle nerve implanted into such a botulinum-poisoned muscle prevents sprouting in the region of the muscle in which the extra nerve forms synapses. If the suppressive effect of the nerve implant on terminal sprouting were simply due to its restoring activity in some of the muscle fibres, as opposed to supplying an antisprouting agent, then direct

electrical activation of a botulinum-paralysed muscle should also prevent terminal sprouting (Brown, *et al.* 1977).

We produced extensive paralysis in both hind legs of five mice by injecting 20 μl. of a solution of unpurified type A botulinum toxin (supplied in this instance by Dr W. E. Van Heyningen, Sir William Dunn School of Pathology: the amount of toxin present was unknown, but (see below) was enough to cause complete paralysis of the soleus). One day later chronic stimulation electrodes were implanted into the right

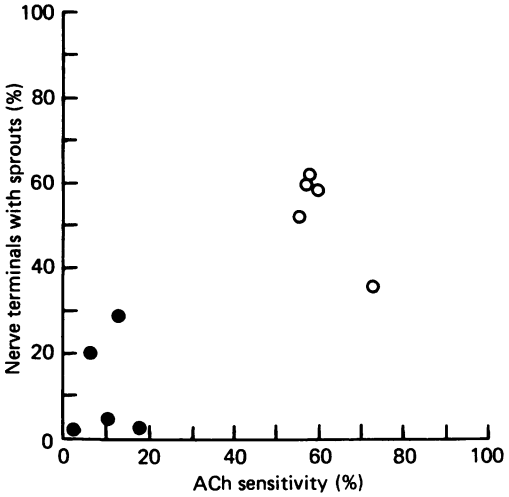

Fig. 1. Effect of direct muscle stimulation on terminal sprouting and ACh sensitivity (contracture tension in 5×10^{-4} g. ml^{-1} ACh perchlorate as percentage of 50 Hz maximal direct tetanic tension) in botulinum-toxin-paralysed soleus muscles. Muscles studied 5–8 days after injection of equal amount of crude type A toxin into the back of both legs. Stimulation was started on one side, after section of the spinal cord at L1 under anaesthesia, one day after toxin injection. ● Stimulated solei; ○ unstimulated.

lower leg and the soleus muscle was directly stimulated, the mice having been spinalized to produce analgesia. Four to six days after the implantation of the wires, the soleus muscles were examined acutely, the left leg muscle serving as an unstimulated control.

Fig. 1 shows the ACh sensitivities and the percentage of terminals with sprouts of the stimulated and unstimulated solei. At the time of acute examination all the muscles were completely blocked (i.e. 50 Hz stimulation of the nerve failed to elicit any tension) and they all would have been expected to show a large sensitivity to ACh (Thesleff, 1960). All the unstimulated muscles did in fact give contractures of more than 50 % of the direct tetanic tension when ACh was applied, but the stimulated muscles were much less sensitive. Hence, the stimulus had been effectively activating most of the muscle fibres in each muscle. All of the control muscles had more than 30 % of their terminals sprouting. The sprouts were usually very fine but often rather extensive, up to 70 μm long. On the other hand the stimulated muscles had much less terminal sprouting, i.e. fewer than 30 % of the end-plates in any muscle were sprouting, and even these were less than 2 μm long. Thus restoring

muscle fibre activity, and presumably normal muscle properties, to this blocked preparation does inhibit the expected terminal sprouting. This, taken with the facts that nerve conduction block by tetrodotoxin (Brown & Ironton, 1977*a*) causes terminal sprouting, and that the post-synaptic blocker α-bungarotoxin does so also (Holland & Brown 1980), strongly supports the concept that inactive or denervated muscle is the source of the stimulus to terminal sprouting and it is not necessary to suppose that botulinum toxin is preventing the release of antisprouting agents.

The production of terminal sprouting by spinalization. Another way to reduce hind-limb neuronal activity without directly interfering with the nerves is to spinalize the animals, and it seemed of interest to see if this operation would cause sprouting. Ten otherwise normal mice had their spinal cords severed (see Methods) and 5–14 days after spinalization one soleus and sometimes the peroneus was isolated and checked for denervation. The muscles examined were all found to be fully innvervated; that is, nerve-evoked tension equalled directly evoked tension, and the number of motor units was normal. Spinalization significantly increases the amount of terminal sprouting in both muscles. In normal solei the percentage of terminals with sprouts is $5{\cdot}5\,\% \pm 0{\cdot}8$, $n = 28$; in solei from spinal mice the figure is $32{\cdot}8\,\% \pm 5{\cdot}9$ $n = 10$, $P < 0{\cdot}001$. In the normal peroneus the percentage of terminals with sprouts is $2{\cdot}1 \pm 0{\cdot}5$, $n = 24$ and in the peronei from spinal mice $26{\cdot}6\,\% \pm 6{\cdot}9$, $n = 5$, $P < 0{\cdot}05$, (means $\pm$ S.E.M. of mean, Student's t test).

In some mice the degree of sprouting was not very marked, but it was difficult to estimate by how much this operation reduced muscle activity. Most mice showed some reflex activity by days 4 and 5 and this may have reduced the effectiveness of the operation as a means of stopping muscle activity.

Relationship between amount of sprouting and amount of block or denervation in different muscles

If the stimulus to terminal sprouting comes solely from inactive muscle fibres (*vide supra*) then on the simplest assumptions the greater the number of inactive fibres the more sprouting might be expected (up to the point of saturation when all terminals are sprouting), and this should apply to muscles paralysed to various extents or denervated by differing amounts.

Again, if nodal and terminal sprouting were the expression of a single influence acting at two different sites on the axon, then one might have expected *a priori:*

(1) to find nodal sprouts in paralysed preparations, but as described previously, neither we (Ironton *et al.* 1978) nor Duchen & Strich (1968) have been able to find nodal sprouts in blocked preparations which have profuse terminal sprouting,

(2) to see a positive correlation between amounts of nodal sprouting and amounts of terminal sprouting. We have noted before (Brown & Ironton, 1978) that soleus more readily produces terminal sprouts after partial denervation than the peroneus tertius. Does the soleus produce more nodal sprouts, too (Brown *et al.* 1978*b*)?

The aim of the experiments described in this section was, therefore, to compare the amounts of nodal and terminal sprouting in different muscles subjected to differing amounts of paralysis or denervation.

Ideally paralysis of part of a muscle would be achieved by applying a tetrodotoxin cuff to one spinal root, but this proved impossible in the mouse. Instead we gave graded doses of botulinum toxin by injection into the hind leg, (0·0001–0·03 μg in volumes of 1–3 μl.), hoping thereby to block completely some end-plates and leave the rest transmitting normally. Fig. 2 shows the directly evoked and nerve-evoked

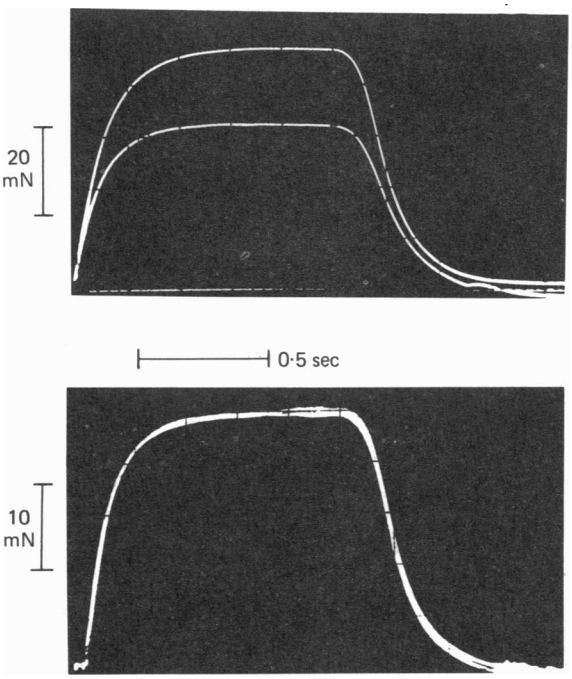

Fig. 2. Incomplete soleus muscle paralysis produced by a single local injection of 0·003 μg in 3 μl. volume of crude type A botulinum toxin into the back of the leg. Above, one directly evoked and one nerve-evoked supramaximal 50 Hz tetanus. Below, three superimposed nerve-evoked 50 Hz tetani. Muscle studied 7 days after injection. Time calibration 0·5 sec.

50 Hz tetanic contraction in a mouse soleus muscle 7 days after a single injection of 0·003 μg of botulinum toxin. Also shown are three superimposed nerve-evoked tetani. Such a dose of toxin does appear to produce a repeatable and unfluctuating degree of block. An initially completely paralysing dose of botulinum toxin remained so in soleus for 10 days and rather longer in peroneus tertius (17 days). We therefore tried to correlate the degree of sprouting with the degree of block between days 6 and 10 in the case of soleus, and 6 and 17 in peroneus tertius, for by day 6 sprouting is clearly present in both muscles.

Differing amounts of partial denervation arose because of the varying distribution of motor axons in roots L4 and L5. Whereas in the botulinum-toxin-paralysed muscles, the degree of paralysis could be expressed by measuring the tension (degree of block = 100 % × (1—nerve-evoked tension/directly evoked tension)), in partial denervation, at times greater than 6 days, nodal and terminal sprouting would have

significantly increased the number of muscle fibres innervated by the remaining axons (Brown & Ironton, 1978) and a different measure of the degree of denervation was required. In each muscle we determined the number of motor units electrophysiologically by graded stimulation of the muscle nerve or ventral root, counting

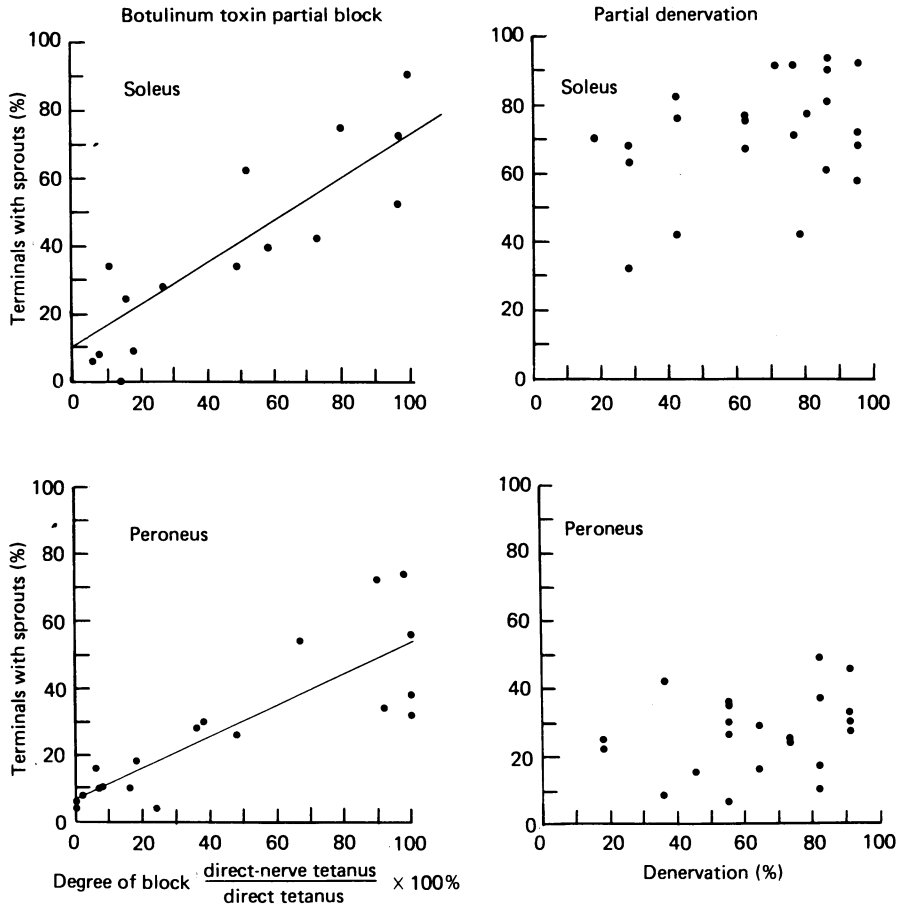

Fig. 3. The relationships between amounts of terminal sprouting and amounts of block or denervation, for soleus and peroneus tertius muscles. The solei were examined 6–10 days post-injection or root section, the peronei at 6–17 days. Note that in the partly denervated muscles four points on the extreme right of the soleus (95 % denervated) and four points on the extreme right of the peroneus (91 % denervated) represent muscles in which only one motor unit remained. Clearly not every terminal sprouts even in these circumstances.

the gradations in tension. On average, using this method the normal peroneus tertius has eleven units and the soleus twenty-one.

Fig. 3 shows the relationship between the amount of paralysis or the amount of denervation and the amounts of terminal sprouting.

In the partly paralysed muscles there were highly significant linear regressions of the proportion of end-plates with terminal sprouts on the degree of block. (soleus: fifteen muscles, $b = 0{\cdot}62$, $P < 0{\cdot}001$; peroneus: nineteen muscles, $b = 0{\cdot}48$, $P < <$

0·001; b = regression coefficient calculated from the method of least squares, P = probability that b differs from zero).

In the partly denervated muscles at the same times, even small amounts of denervation caused nearly as large amounts of terminal sprouting as greater denervations, and the regression line slopes did not differ significantly from zero.

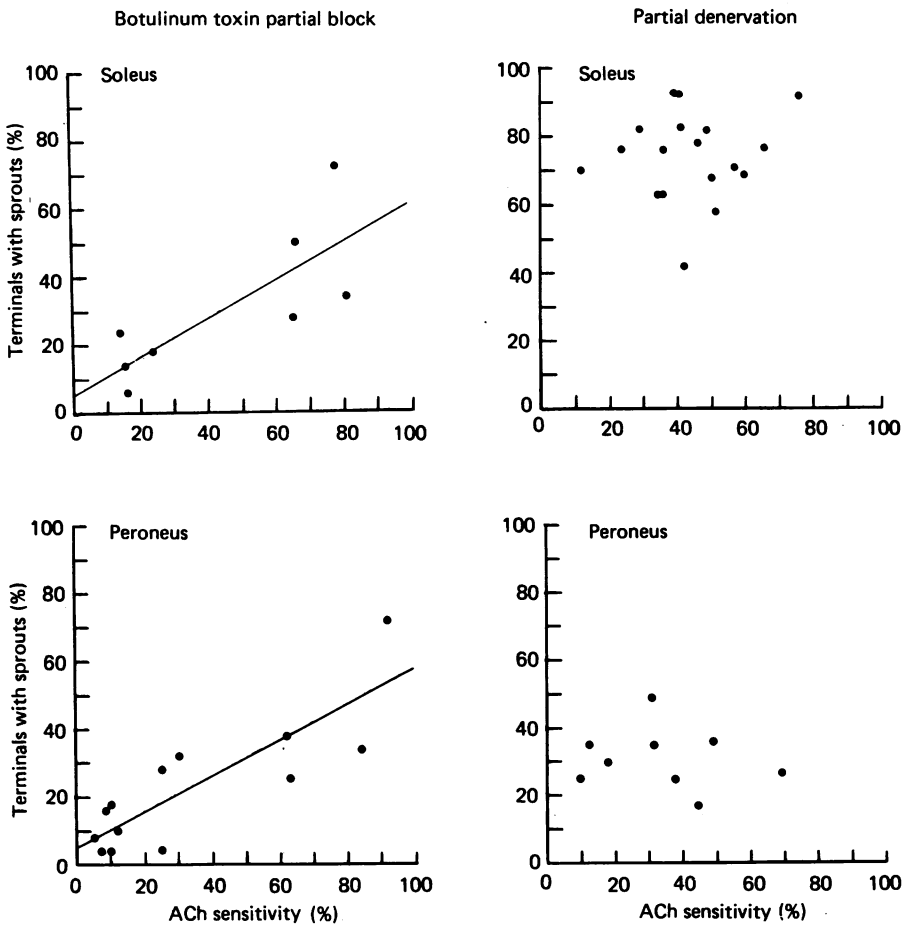

Fig. 4. Relationships between amounts of terminal sprouting and ACh sensitivities for partly paralysed and partly denervated soleus and peroneus tertius muscles.

The ACh sensitivities of some of the partly blocked and partly denervated muscles were also measured and the relationships between the amount of sprouting and these sensitivities are shown in Fig. 4. The ranges of ACh sensitivities achieved are much the same for the two classes of preparation. This demonstrates that similar ranges of muscle inactivity were also achieved by the two procedures. In the blocked preparations there were significant linear regressions of the proportion of end-plates with terminal sprouts on the ACh sensitivity (soleus, $n = 8$, $b = 0{\cdot}56$, $P < 0{\cdot}05$; peroneus, $n = 13$, $b = 0{\cdot}53$, $P < 0{\cdot}001$). There was no such correlation in the partly denervated preparations.

The lengths of the sprouts at different degrees of block were also measured. In the peroneus, although there was a wide range of sprout sizes in any one muscle, there was very little variation in the mean sprout lengths of different muscles whether the muscles had been very weakly or strongly blocked. The average sprout length for all muscles was 23 μm $\pm$ 5·4 (S.E. of mean), n = 17. The same general observations were true of the soleus except that in four muscles where the block was less than 15 % the sprouts were only on average about 11 μm, whereas at all other degrees of block from 20 to 100 % the average length was higher (mean for all muscles 29·3 μm $\pm$ 6·1 μm, n = 8). These results reinforce our belief that end-plates were either blocked or not blocked over a wide range of doses of toxin, and that recovery from small doses of toxin, for the individual end-plates involved, is not necessarily faster than it is when larger doses are administered, a factor which might have distorted the comparison between the blocked preparations.

The rather linear and regular relationship between the amount of terminal sprouting and degree of block and ACh sensitivity in the preparations paralysed to various extents accords well with the idea that the inactive muscles are the source of the stimulus and in addition suggests that there is no great excess of this factor available; otherwise, for example, a half-paralysed preparation might be expected to have considerably more than half of its terminals sprouting.

The independence of the amount of terminal sprouting and the presumed number of inactive muscle fibres in the partly denervated preparations is surprising. The implication is that there is an additional factor present in denervated muscles which has an effect on terminal sprouting. We defer discussion of this until later.

Inspection of Fig. 3 also shows that in soleus the amount of terminal sprouting when the block is nearly complete (80 % or more) is equal to the amount of terminal sprouting in the partly denervated muscles. In peroneus the average amount of terminal sprouting in muscles 80 % or more blocked is 52 %; this is not significantly different from the comparable figure for blocked soleus which is 72 % (Mann-Whitney U test $0{\cdot}09 < P < 0{\cdot}13$). However the average amount for peroneal muscles 80 % or more denervated is only 31 %. This is significantly less (Mann–Whitney U test $P < 0{\cdot}025$) than the figure for the blocked peroneal muscles. Thus although the fast peroneus tertius terminals are probably potentially as able to sprout as those in soleus, they do not do so in a partly denervated muscle. This phenomenon is also clearly seen in the gluteus muscle, which we have investigated with Dr W. G. Hopkins. We find that when this muscle is partly denervated fewer than 40 % of terminals sprout, and usually fewer than 20 %. But in four muscles injected with 0·01 μg in 1 μl. of botulinum toxin more than 60 % of terminals sprouted.

Fig. 5 gives the percentage of end-plates in soleus and peroneus tertius muscles innervated by nodal sprouts after partial denervation. As in the case of terminal sprouts, there is no significant correlation between the amount of denervation and the amount of nodal sprouting measured in this way.

Our estimate of the amount of nodal sprouting is not of course absolute, i.e. it does not estimate the percentage of intramuscular nodes producing sprouts. It probably counts, however, a large proportion of the nodal sprouts which are visible under the light microscope, for (Slack, Hopkins & Williams, 1979) it turns out that at whatever time a partly denervated preparation is examined, 80 % of nodal sprouts have

terminated on muscle fibres, presumably because once stimulated to grow they cover the relatively small distances in a short time (see also Hoffman, 1950).

At all events it is justifiable to use our measure of nodal sprouting to compare the *relative* amounts of nodal sprouting in the two muscles and it is clear from Fig. 5 that

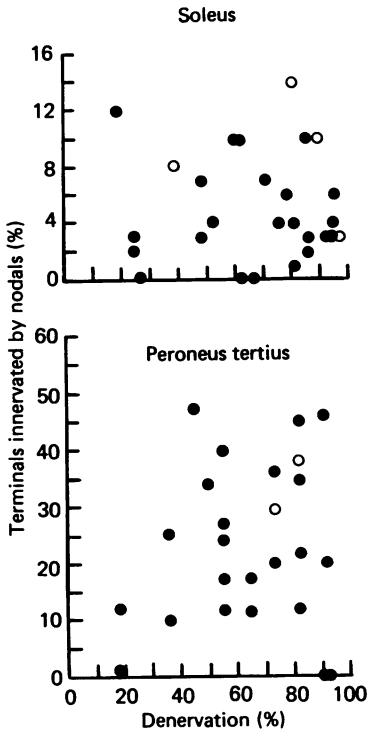

Fig. 5. The relationship between the percentage of end-plates innervated by nodal sprouts and the amount of denervation in the soleus and peroneus tertius muscles. Filled circles examined prior to 11 days post-operation, open circles examined after 11 days. Note different scales for ordinates for the two muscles. The regression line for the soleus (6–10 days) is $y = 4{\cdot}2 + 0{\cdot}00\,x$. N.S. The regression line for the peroneus (6–17 days) is $y = 17{\cdot}9 + 0{\cdot}1\,x$. N.S.

peroneus has more than soleus. There thus appears to be a reciprocal relationship between fast and slow muscles in response to being partly denervated. The slow soleus has relatively less nodal sprouting. The results from investigations of the extensor digitorum longus muscle confirm these points. This fast muscle produces even less terminal sprouting than peroneus tertius (mean $22{\cdot}5\,\% \pm 3{\cdot}1$ S.E. of mean, $n = 7$) and more nodal sprouting ($33{\cdot}6\,\% \pm 5{\cdot}9$, $n = 7$).

These semi-quantitative observations suggest not only that in partly denervated muscles there may be sprout-inducing agents present in addition to denervated muscle fibres, but also illustrate the differences to be found between fast and slow muscles. An explanation has to be found for the greater similarity between fast and slow muscles when transmission is blocked than when they are partly denervated.

Effect of direct muscle stimulation on nodal sprouting

It is now clear (Brown & Ironton, 1977*b*; Ironton *et al.* 1978; Brown & Holland, 1979) that the amount of terminal sprouting in partly denervated muscles is dramatically reduced by direct stimulation of the muscles. We reported (Ironton *et al.* 1978) that nodal sprouting was reduced by such stimulation but not significantly. The comparison of the nodal sprouting was made largely between partly denervated

TABLE 1. Effect of direct muscle stimulation on the effectiveness of nodal and terminal sprouts in forming end-plates. Muscles studied 4–18 days after partial denervation. Mean time for soleus 7·9 days, for peroneus 7·7 days. All data from spinal preparations. Figures are ± S.E. of mean.

	Soleus			Peroneus tertius		
	Unstimulated	Stimulated	*P*	Unstimulated	Stimulated	*P*
% terminals innervated by nodal sprouts	3·3 ± 1·1 *n* = 13	2·36 ± 0·9 *n* = 11	N.S.	6·2 ± 2·8 *n* = 10	10·2 ± 2·3 *n* = 8	N.S.
% terminals innervated by terminal sprouts	8·4 ± 5·8 *n* = 13	0·6 ± 0·4 *n* = 11	< 0·001	8·3 ± 3·5 *n* = 10	2·3 ± 0·72 *n* = 8	N.S.

muscles from normal mice and the stimulated muscles from mice whose spinal cords had been cut in the low thoracic or high lumbar region so that stimulation could be carried out without hurting the animals. Spinalization, possibly because the animals are in less good general health, does appear to reduce the amount of nodal and terminal sprouting when a muscle is partly denervated (Ironton *et al.* 1978). Indeed such a non-specific reduction can be seen on comparing the data of Table 1 with that of Fig. 5. Therefore, the correct comparison should be between partly denervated muscles in spinal preparations and similarly partly denervated muscles in spinal preparations whose muscles have been kept active by direct stimulation.

In Table 1, we present the re-analysed data. The percentage of terminals supplied by nodal sprouts remains much the same in the stimulated muscles as in the unstimulated ones. In contrast, the percentage of terminals supplied by branches from other terminals is very significantly reduced in soleus and also reduced in peroneus although not significantly.

Absence of a central signal to sprouting in mouse muscle

The possibility of a central source of sprouting stimulus has been raised by the experiments of Rotshenker & McMahan (1976) and Rotshenker (1979), where in the frog cutaneous pectoris muscle unilateral denervation increased the percentage of multiple innervation in the contralateral muscle. It is suggested that the biochemical change that causes the cut axon of a motoneurone to grow can spread from the axotomized and chromatolysing motoneurones to affect neighbouring neurones. Can it do so in mice (Brown *et al.* 1979)?

Complete sciatic nerve section in one leg was carried out and caused no significant sprouting in the soleus and peroneus tertius muscles of the other leg which were examined 4–24 days after the operation. The mean percentage of terminals with sprouts was 5·8 ± 1·4, $n = 12$ for the soleus and 5·4 ± 2·3, $n = 12$ for the peroneus.

(Normal values are $5{\cdot}5 \pm 0{\cdot}8$, $n = 28$ for the soleus and $2{\cdot}1 \pm 0{\cdot}5$, $n = 24$ for the peroneus). To bring chromatolysing motoneurones closer to the normal ones in the cord we also denervated all but the soleus muscle in one lower leg by cutting the muscle nerves in the popliteal fossa; sham operations were done on the opposite leg in some animals. This operation did cause a just significant ($P < 0{\cdot}05$) rise in the

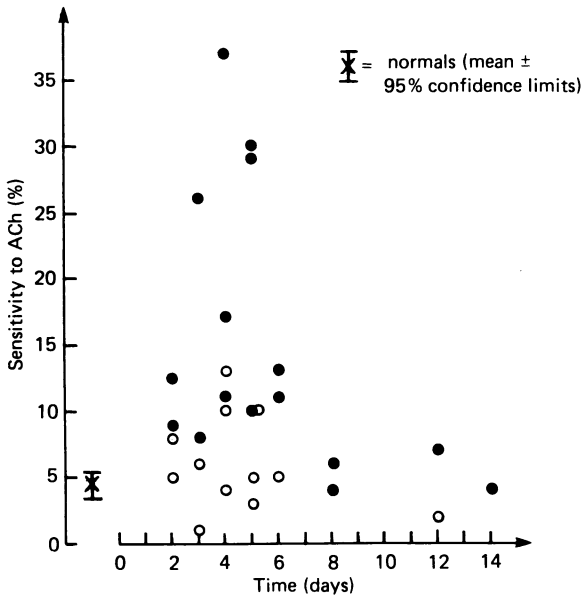

Fig. 6. Acetylcholine sensitivity in soleus muscles at different times after denervating all but the soleus in the lower leg (●) or after sham operation (○).

percentage of terminals with sprouts in the test solei when compared with normal muscles (normals $5{\cdot}5 \pm 0{\cdot}8$, $n = 28$; test solei $9{\cdot}2 \pm 1{\cdot}1$, $n = 12$). However, the sham operated mice also showed a rise ($6{\cdot}5 \pm 1{\cdot}0$, $n = 7$) and the difference between the sham and experimental muscles is not significant. In any case the increase in sprouting is minimal when compared with the effects of partial denervation. Neither contralateral denervation nor deneveration of muscles around the soleus caused any nodal sprouting.

We did find (Fig. 6) that the ACh sensitivities of the solei were transiently raised (days 2–6) by the surround denervation operation, and also in sham operated mice, test muscles significantly more than sham muscles (contracture tensions of $18\% \pm 2{\cdot}9$, $n = 12$, tests; $6{\cdot}4\% \pm 1{\cdot}1$, $n = 11$, shams; $4{\cdot}5\% \pm 0{\cdot}6$, $n = 20$, normals). Thus these operations, particularly those in which there was massive denervation in the leg, caused a peripheral change in the soleus. This change could itself explain the small increase in terminal sprouting following a 'surround denervation' operation, and there seems no need to postulate a central signal. The relatively small rise in ACh sensitivity is interesting in demonstrating how well insulated individual muscles appear to be from one another as far as sensitivity-inducing agents go.

It has been reported (Greenman (1913), Nittono (1922), Tamaki (1933, 1936) that cutting a nerve on one side in mammals may cause neuronal degeneration in the

contralateral nerve. This effect is apparently pronounced at early times after the operation (4 days) and in young or neonatal animals. In our contralateral denervations we found no evidence of such crossed degeneration, even in four young mice only 3–5 days after operation, in which careful estimates of the numbers of remaining motor units were made.

From these results it does not appear that a central signal can, on its own, cause sprouting in mammalian preparations. We also have evidence against the idea that sprouting involves a complete switch on of individual motor units. Reference to Fig. 3 shows that when terminals of single motor units are studied, which is possible when only a single motor unit is left behind after partial denervation, not all of them sprout. This implies that the terminals behave independently, or that there is a maximum capacity for growth of the motoneurone as a whole, the nerve being able to direct its growth effort into selected terminals.

DISCUSSION

Methods

Before trying to reach conclusions from the present experiments it is necessary to consider the methods. Our measurement of sprouting is done entirely from teased bundles of muscle fibres stained with zinc iodide and osmium. Teasing naturally will destroy a certain proportion of the nerves and end-plates, and it might be argued, that this would be more likely to affect nodal sprouts. It might also be argued that the ZIO stain, although undoubtedly the method of choice for revealing terminal sprouts, may be rather poor at detecting nodal sprouts. Thus, our figures for the amount of nodal sprouting may be on the low side, and as pointed out earlier are in no sense 'absolute' anyway, but there seems no reason to suppose that the relative amounts of nodal sprouting in different muscles should be wrongly estimated. Preliminary results suggest in any case that the ZIO method probably does not seriously underestimate nodal sprouting when the method is compared with a silver impregnation technique (authors' personal observation).

The index used for sprouting has been simply to express the number of end-plates with terminal sprouts, the number innervated by terminal sprouts and the number innervated by nodal sprouts, as a percentage of the total number of end-plates examined. It would be expected that the muscles with greater amounts of denervation would inevitably have more sprouting on this index, for fewer of the end-plates could be normal and more would be available for innervation by sprouts of both sorts. In Figs. 3 and 5 it can however be seen that there is only a slight and statistically insignificant tendency for greater amounts of denervation to be associated with more sprouting. One reason for this surprising result may lie in the observation made above, that when single motor units are studied not all terminals sprout. It is also clear from inspection of silver-impregnated whole mounts of partly denervated muscles that only a proportion of the intramuscular nodes become involved even in extensively denervated preparations (Slack, Hopkins & Williams, 1979; authors' observations). Another possibility which should be borne in mind is that in lumping together data from muscles over a time span of up to 11 days a significant degree of maturation may have occurred, so that the earliest nodal sprouts become myelinated

and indistinguishable from normal intramuscular branching, and excess terminal sprouts might retract as activity is restored. It is unlikely, however, that this can explain the results, for when the data of Figs. 3 and 5 were analysed in narrower time bins we again failed to find a significant correlation between amounts of denervation and sprouting.

The third methodological problem is the way in which we attempted to produce a partial block of the muscle. Fig. 2 shows that a muscle treated with a small dose of botulinum toxin can give a non-sagging reproducible tetanic contraction which is a fixed percentage of the total muscle tension. What is not clear is whether some terminals are not fully blocked. A large proportion of partly blocked fibres might not necessarily produce a noticeable fluctuation in tetanic tension. Presumably, however, the extent of denervation-like changes in a partly blocked muscle fibre would be related to the degree of block. It follows that for the muscle as a whole correlations between terminal sprouting, degree of block and ACh sensitivity would be expected if muscle fibre changes are causally related to the sprouting stimulus.

A fourth problem with our methods is whether direct stimulation of muscles with currents of up to 40 mA inhibits sprouting by non-specific means, for example by destroying fine nerve outgrowths. In work published elsewhere (Brown & Holland, 1979) we have shown that this is very unlikely as muscle re-innervation following complete nerve crush (a process in which fine nerve outgrowths are also involved) is not inhibited by currents of equal magnitude delivered in exactly the same manner.

Terminal sprouting. The production of terminal sprouting in the muscles of spinal mice in which activity is clearly decreased substantially (Solandt & Magladery, 1942), and the prevention of sprouting in botulinum-poisoned muscles by direct activation, add further support to the view (Brown & Ironton, 1977*a*; Holland & Brown, 1980) that inactive muscle is the source of the stimulus for this form of sprouting. We have previously suggested that the stimulus might not be a diffusible growth factor liberated from the muscle, but rather a change in the surface of the muscle fibres which permits an inherent sprouting tendency to manifest itself (Brown *et al.* 1978*a*). We explained the ability of the intact terminals in partly denervated muscles to sprout on this model on the grounds that the innervated fibres in such a muscle also show surface changes (Cangiano & Lutzemburger, 1977; Brown, Holland & Ironton, 1978*c*). There are three reasons why this suggestion should be reconsidered.

First the idea was based on the linearity of the relationship between amount of block and amount of terminal sprouting, together with the fact that sprouts in such partly blocked muscles occurred usually on the smaller (hence paralysed and atrophied) fibres. From the points raised above about the difficulty of assessing how 'effective' a partial block can be produced with submaximal botulinum toxin injections, it would be unwise to use this as a major argument in favour of the idea. Secondly, we (Brown & Holland, 1979) have shown that prevention of terminal sprouting in stimulated partly denervated muscles is due, not to activation of the innervated fibres with consequent suppression of the surface changes on them, but to activation of the denervated fibres. Thus the signal must be detectable by the intact terminals at a distance. Thirdly, Betz, Caldwell & Ribchester (1980) in an elegant experiment making use of muscles in the rat foot, which are supplied by two well separated nerves, have shown that if one nerve is blocked with a tetrodotoxin cuff, the unblocked terminals innervated by the other nerve do develop terminal sprouts.

Does this mean that inactive and denervated muscle fibres liberate a 'motor nerve growth factor'? That is certainly one possibility. Alternatively it might be that nerve terminals are normally probing their environment with outgrowths not visible with the light microscope. When these detect a suitable surface they might attach, grow further and enlarge to become visible with conventional staining methods.

Nodal sprouting. The evidence that nodal sprouting is triggered independently of terminal sprouting can be summarized as follows:

1. nodal sprouts have not been seen in paralysed preparations which have terminal sprouts (Duchen & Strich, 1968; Ironton *et al.* 1978);
2. nodal sprouting is not prevented by direct muscle stimulation which reduces terminal sprouting (Ironton *et al.* 1978) and the present paper;
3. the data presented here which show that the relative amounts of nodal and terminal sprouting are not necessarily naturally correlated in partial denervation—a slow muscle will show relatively more terminal and relatively less nodal sprouting than a fast muscle.

As the following discussion will show, this evidence is however by no means conclusive. The failure to see nodal sprouts in paralysed muscles could be due to a variety of other reasons. The intact and nerve-filled perineural sheaths in blocked muscles might act as a barrier to material liberated from the muscle, a consideration that might not apply to such agents liberated in a partly denervated muscle. Alternatively, blocked muscles might release too little compared with denervated muscles or the sprouts might be present but not visible at the light microscope level in the absence of a suitable surface to grow on or target to innervate. Possibly excess terminal sprouting might inhibit nodal sprouting in some way. However we can probably exclude the idea that nerve blockers like tetrodotoxin or botulinum toxin prevent nodal sprouting by stopping neuronal uptake and retrograde transport of a muscle released sprouting agent, for partly denervated peroneus muscles which we treated with botulinum toxin had an average of 17% of end-plates innervated by nodal sprouts, not far from the normal average of 23%.

Again, if nodal sprouting were due solely to nerve degeneration products it is hard to see why nodes all along the length of axons exposed to degenerating fibres do not sprout (Edds, 1949). Hoffman was aware of this problem, and in a very early electron microscope study (Causey & Hoffman, 1955) maintained that extramuscular nodal sprouting occurred profusely. The definition obtained in these pioneering pictures is very poor and we are in the process of re-examining this problem.

The fact that we do not suppress the number of successful nodal sprouts by direct muscle stimulation, whereas the percentage of terminals with sprouts is dramatically reduced (Brown & Ironton, 1977*b*; Ironton *et al.* 1978; Brown & Holland, 1979), must also be considered together with two points which somewhat reduce its use as a powerful argument for separate stimuli for nodal and terminal sprouting. First, at the times we inspect the muscles which are limited by how long we can continue stimulation, the number of successful nodal sprouts identified by us in spinal preparations is anyhow rather small. Secondly, although the number of successful *terminal* sprouts was significantly reduced in the soleus the reduction that occurred in the peroneus was not significant.

Differences between fast and slow muscles in sprouting pattern

The 'extra' factor needed to explain the larger amount of terminal sprouting in a lightly denervated soleus compared with a lightly blocked one is probably the so-called 'inflammatory effect' of nerve degeneration products on muscle fibre properties, which increases the rate of development of denervation effects in denervated fibres (Lømo & Westgaard, 1976) and causes denervation-like changes to develop on innervated fibres (Jones & Vrbova, 1974; Cangiano & Lutzemburger, 1977; Brown *et al.* 1978*c*). The agent responsible for this effect is unknown but it clearly spreads effectively in muscle and could enhance terminal sprouting: (1) by causing the innervated fibres to generate a sprouting signal, (2) by augmenting the inactivity induced changes on the denervated fibres and causing them to liberate more sprouting agent, (3) by acting directly on the nerve terminals.

'Inflammatory effects' are less able to change muscle properties in fast muscles (Lømo & Westgaard, 1976) and this could help to explain why the partly denervated peroneus tertius is less susceptible to its terminal sprout-inducing effects.

Fast muscles also take some days longer than slow muscles to undergo denervation changes (Lømo & Westgaard, 1976) and this will also slow the appearance of the stimulus to terminal sprouting. In the meantime nodal sprouts may have begun to reactive denervated fibres and cut off the possibility of further change. When a fast muscle has its terminals blocked for long periods, however, the muscle changes inevitably eventually appear and terminal sprouting will arise. In this way the greater amount of terminal sprouting in a heavily paralysed fast muscle than in an extensively denervated one can be explained.

Why the soleus has relatively less nodal sprouting than the peroneus is unclear. Perhaps nodal and terminal sprouting compete for a limited amount of a common ingredient required for growth cone production. The large amounts of terminal sprouting found in partly denervated soleus muscles might thus inhibit nodal sprouting.

Conclusions. There seems to be no evidence in favour of a central signal to sprouting in mouse motoneurones. Peripheral signals, of which one certainly arises in the denervated muscle, and another possibly from degenerating nerves (Hoffman, 1950; Hoffman & Springell, 1951; Brown, *et al.* 1978*c*) could account for terminal and nodal sprouting respectively. Further work is needed to confirm that the two forms of sprouting are indeed elicited by two different means, and to find out what these are.

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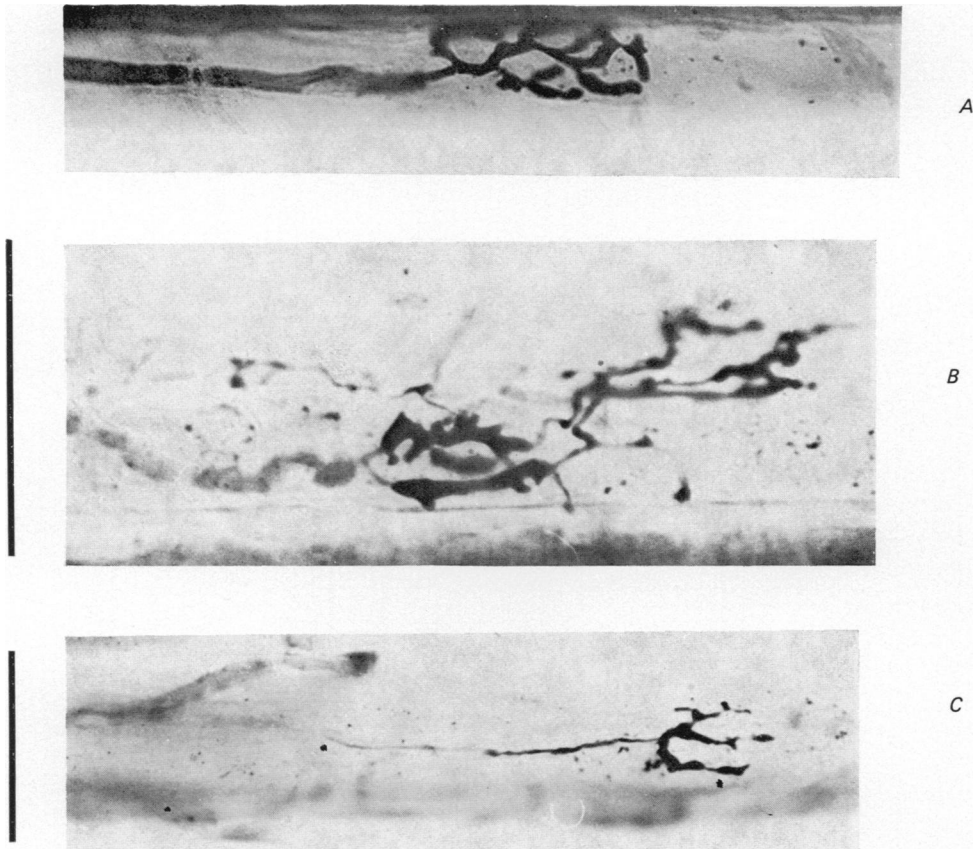
EXPLANATION OF PLATE 1

End-plates from mouse muscles stained with zinc iodide and osmium tetroxide to show appearance of sprouts.

A, normal soleus end-plate. *B*, terminal sprouting in soleus 9 days after partial denervation by section of L4 ramus. Soleus had only one motor unit left. *C*, nodal sprout in peroneus tertius 8 days after section L4 ramus. Myelinated axon from which nodal sprout arises can be seen running diagonally across the left top hand corner of the micrograph. Calibration bars are 50 μm; top applies to *A* and *B*; bottom to *C* only.


A
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